Title: RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING
Docket No.: 37851-923
Applicant: Gantier et al.
Filed: September 8, 2003

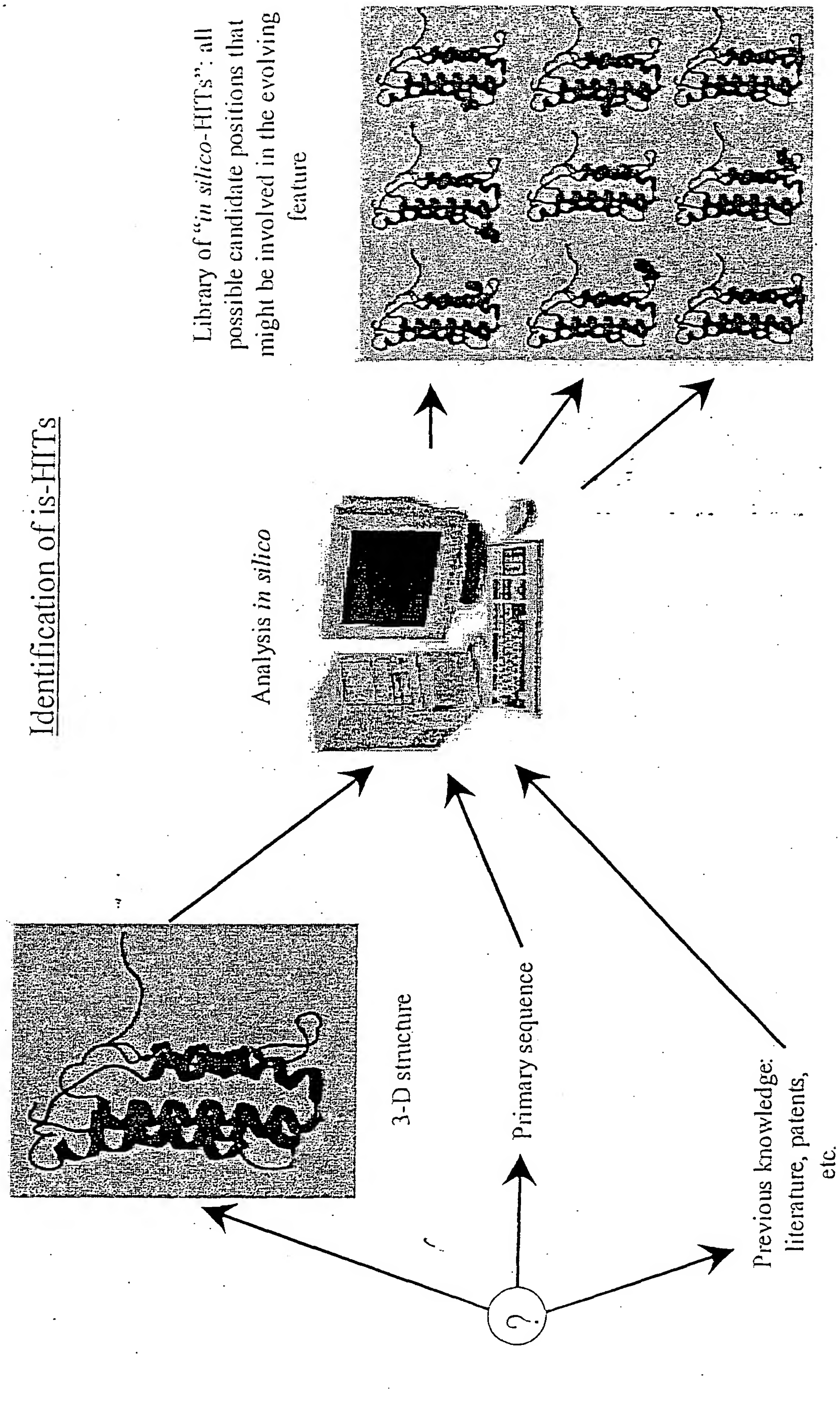

FIG.1A

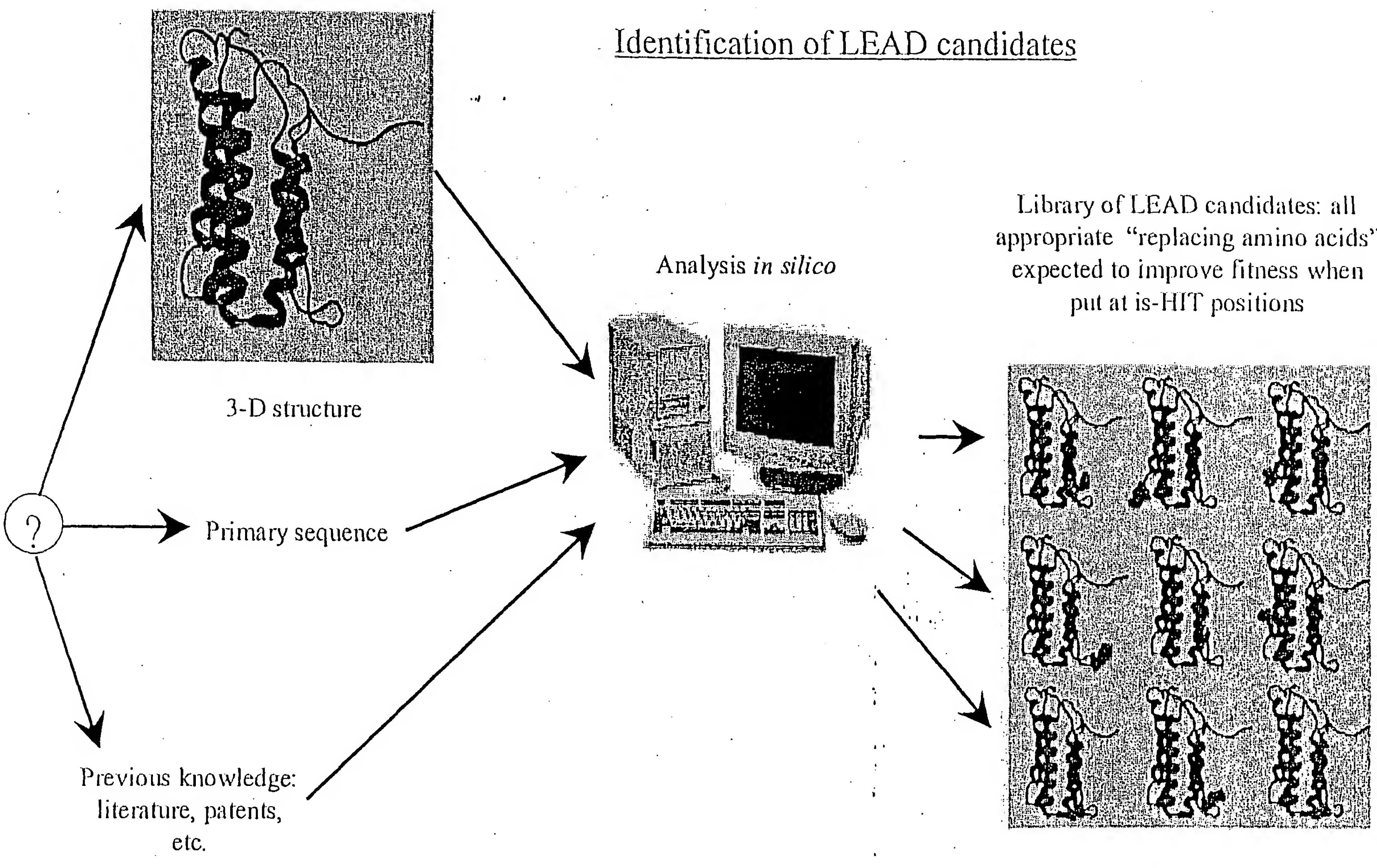
Identification of LEAD candidates
3-D structure
?
Primary sequence
Previous knowledge: literature, patents, etc.
Analysis *in silico*
Library of LEAD candidates: all appropriate "replacing amino acids" expected to improve fitness when put at is-HIT positions

FIG.1B

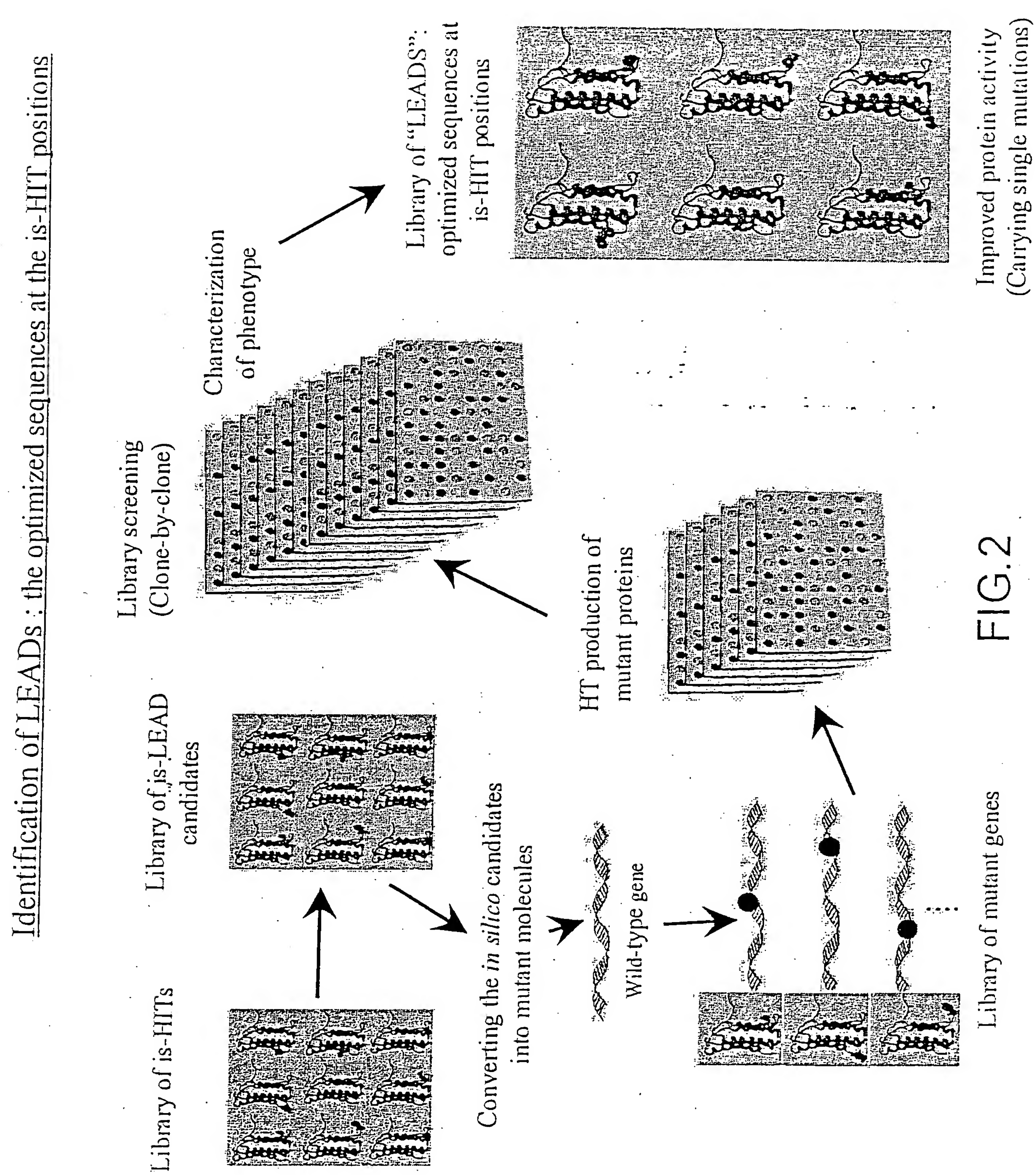
Identification of LEADs : the optimized sequences at the is-HIT positions
Library of is-HITs
Library of is-LEAD candidates
Converting the *in silico* candidates into mutant molecules
Wild-type gene
Library of mutant genes
HT production of mutant proteins
Library screening (Clone-by-clone)
Characterization of phenotype
Library of "LEADS": optimized sequences at is-HIT positions
Improved protein activity (Carrying single mutations)

FIG.2

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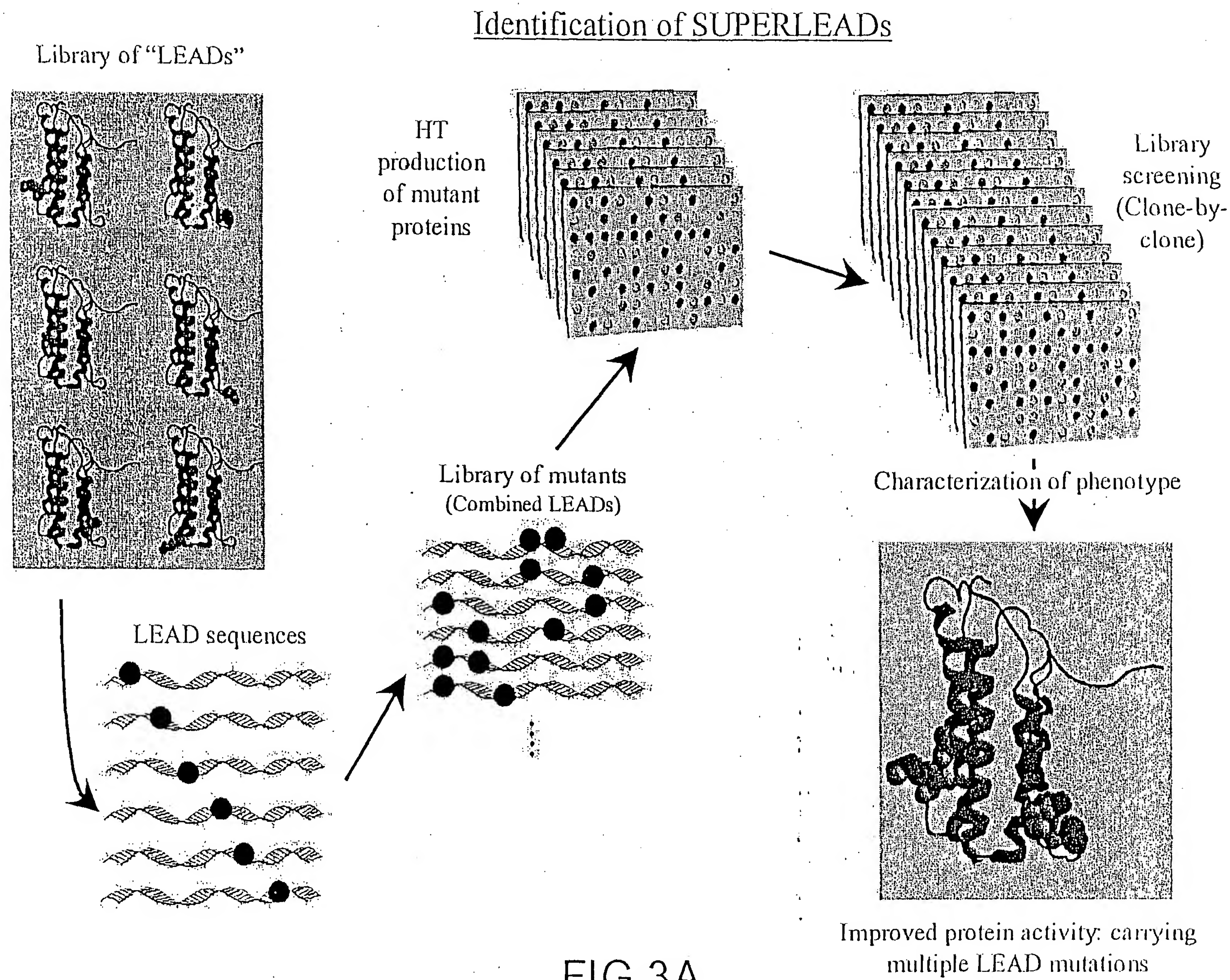

FIG.3A

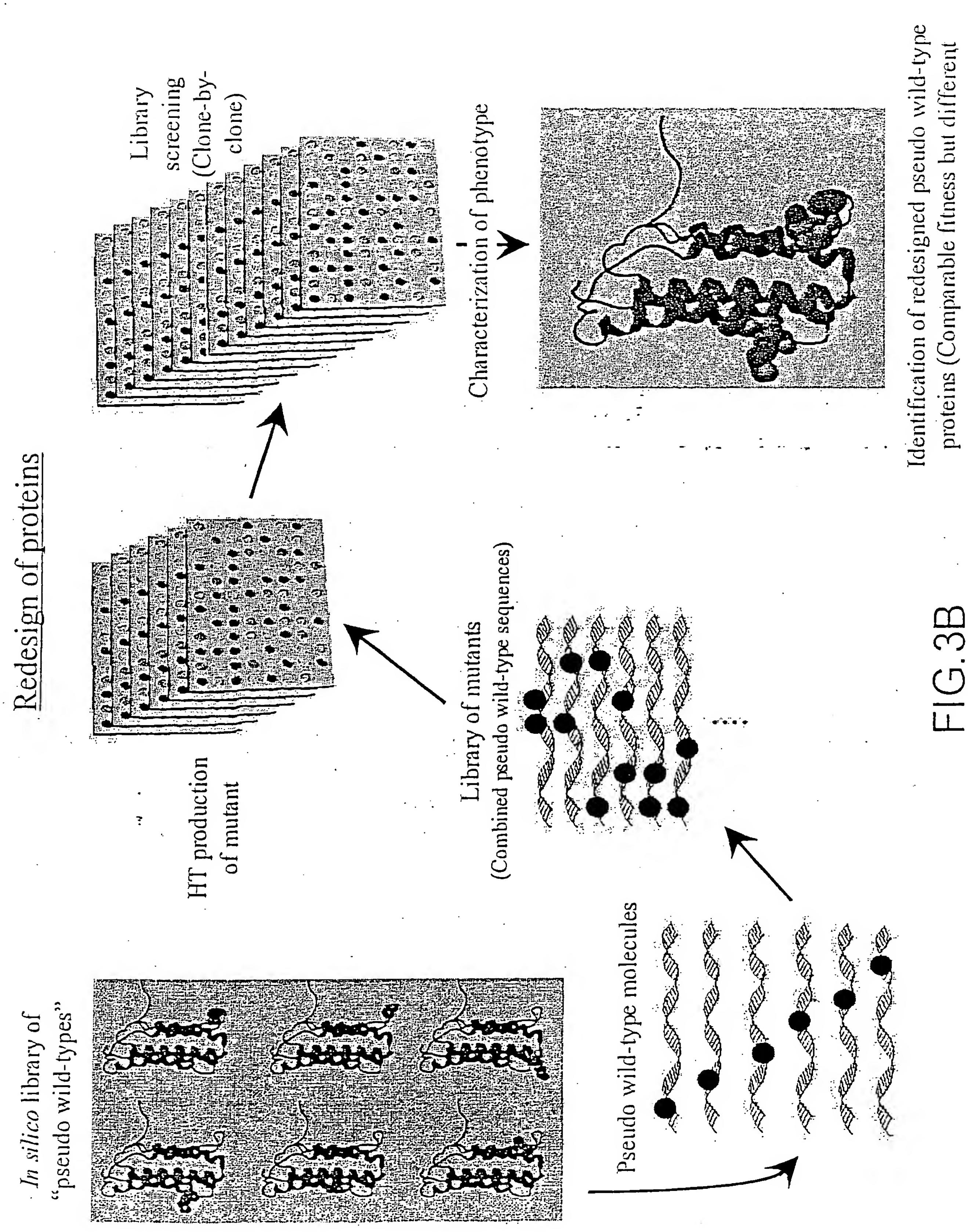
Redesign of proteins
In silico library of "pseudo wild-types"
Pseudo wild-type molecules
Library of mutants
(Combined pseudo wild-type sequences)
HT production of mutant
Library screening (Clone-by-clone)
Characterization of phenotype
Identification of redesigned pseudo wild-type proteins (Comparable fitness but different sequence compared to the wild-type)

FIG.3B

Title: RATIONAL DIRECTED PROTEIN EVOLUTION USING TWO-DIMENSIONAL RATIONAL MUTAGENESIS SCANNING
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"Additive Directional Mutagenesis" (ADM)

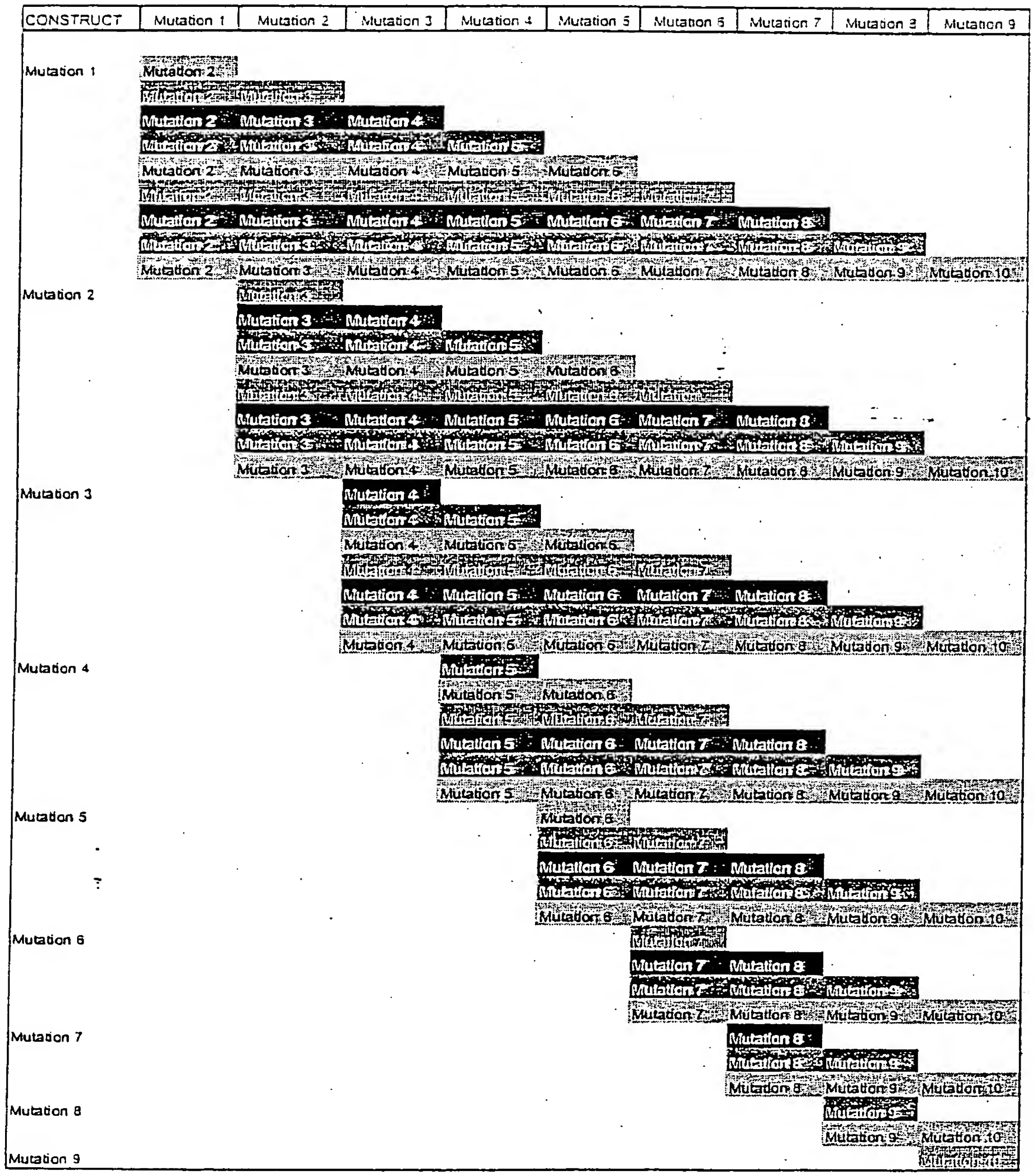

FIG.4

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LEADs and SuperLEADs obtained for the Rep protein

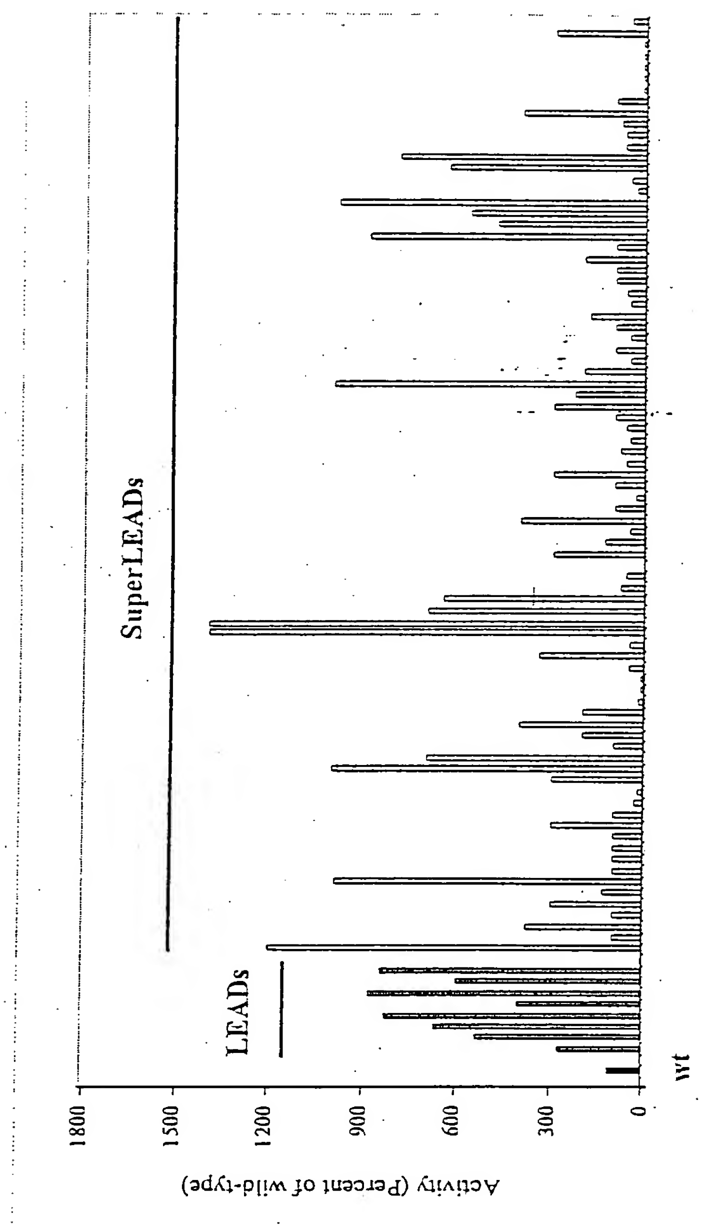

FIG.5

Amino acid sequence of human mature IFNα-2b

	1	10	20	30	40	50
IFNα-2b	CDLPQTHSLGSRRTLMLLAQMRRISLFSCLKDRHDFGFPQEEFGNQFQKA					
	51	60	70	80	90	100
IFNα-2b	ETIPVLHEMIQQIFNLFSTKDSSAAWDETLLDKFYTELYQQLNDLEACVI					
	101	110	120	130	140	150
IFNα-2b	QGVGVTETPLMKEDSILAVRKYFQRITLYLKEKKYSPCAWEVVRAEIMRS					
	151	160				
IFNα-2b	FSLSTNLQESLRSKE					

FIG.6A

Three dimensional structure of INFα-2b

showing candidate LEADs

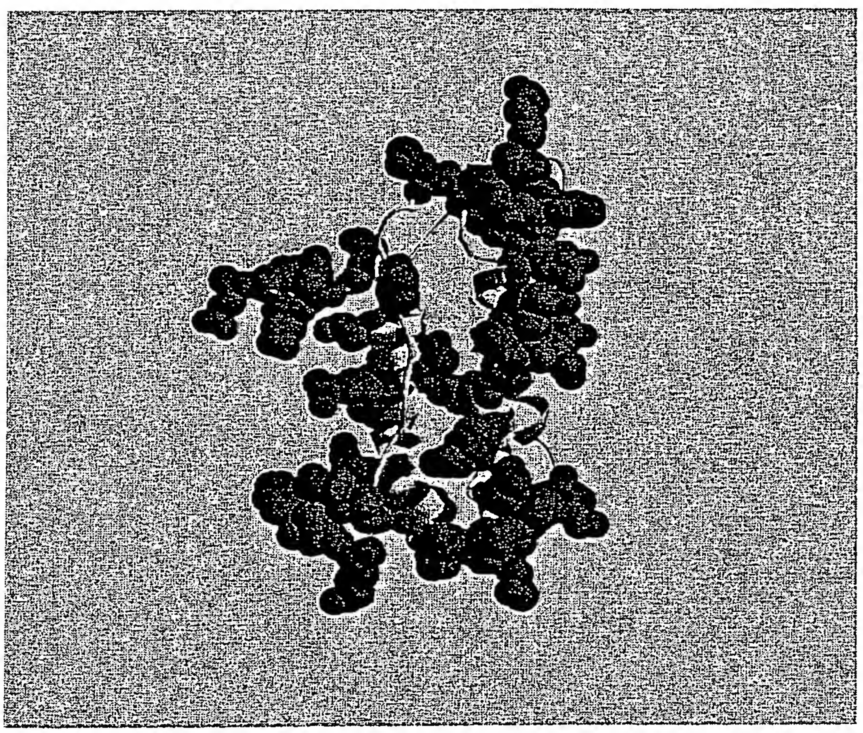

FIG.6B

The "Percent Accepted Mutation" (PAM250) matrix

	A	R	N	D	C	Q	E	G	H	I	L	K	M	F	P	S	T	W	Y	V
A	2	-2	0	0	-2	0	0	1	-1	-1	-2	-1	-1	-3	1	1	1	-6	-3	0
R	-2	6	0	-1	-4	1	-1	-3	2	-2	-3	3	0	-4	0	0	-1	2	-4	-2
N	0	0	2	2	-4	1	1	0	2	-2	-3	1	-2	-3	0	1	0	-4	-2	-2
D	0	-1	2	4	-5	2	3	1	1	-2	-4	0	-3	-6	-1	0	0	-7	-4	-2
C	-2	-4	-4	-5	12	-5	-5	-3	-3	-2	-6	-5	-5	-4	-3	0	-2	-8	0	-2
Q	0	1	1	2	-5	4	2	-1	3	-2	-2	1	-1	-5	0	-1	-1	-5	-4	-2
E	0	-1	1	3	-5	2	4	0	1	-2	-3	0	-2	-5	-1	0	0	-7	-4	-2
G	1	-3	0	1	-3	-1	0	5	-2	-3	-4	-2	-3	-5	0	1	0	-7	-5	-1
H	-1	2	2	1	-3	3	1	-2	6	-2	-2	0	-2	-2	0	-1	-1	-3	0	-2
I	-1	-2	-2	-2	-2	-2	-2	-3	-2	5	2	-2	2	1	-2	-1	0	-5	-1	4
L	-2	-3	-3	-4	-6	-2	-3	-4	-2	2	6	-3	4	2	-3	-3	-2	-2	-1	2
K	-1	3	1	0	-5	1	0	-2	0	-2	-3	5	0	-5	-1	0	0	-3	-4	-2
M	-1	0	-2	-3	-5	-1	-2	-3	-2	2	4	0	6	0	-2	-2	-1	-4	-2	2
F	-3	-4	-3	-6	-4	-5	-5	-5	-2	1	2	-5	0	9	-5	-3	-3	0	7	-1
P	1	0	0	-1	-3	0	-1	0	0	-2	-3	-1	-2	-5	6	1	0	-6	-5	-1
S	1	0	1	0	0	-1	0	1	-1	-1	-3	0	-2	-3	1	2	1	-2	-3	-1
T	1	-1	0	0	-2	-1	0	0	-1	0	-2	0	-1	-3	0	1	3	-5	-3	0
W	-6	2	-4	-7	-8	-5	-7	-7	-3	-5	-2	-3	-4	0	-6	-2	-5	17	0	-6
Y	-3	-4	-2	-4	0	-4	-4	-5	0	-1	-1	-4	-2	7	-5	-3	-3	0	10	-2
V	0	-2	-2	-2	-2	-2	-2	-1	-2	4	2	-2	2	-1	-1	-1	0	-6	-2	4

FIG.7

Scores from PAM250, given to residue substitutions to protect human INF α−2b against proteolysis

	R	D	E	L	K	M	F	P	W	Y
A	-2	0	0	-2	-1	-1	-3	1	-6	-3
N	0	2	1	-3	1	-2	-3	0	-4	-2
C	-4	-5	-5	-6	-5	-5	-4	-3	-8	0
Q	1	2	2	-2	1	-1	-5	0	-5	-4
G	-3	1	0	-4	-2	-3	-5	0	-7	-5
H	2	1	1	-2	0	-2	-2	0	-3	0
I	-2	-2	-2	2	-2	2	1	-2	-5	-1
S	0	0	0	-3	0	-2	-3	1	-2	-3
T	-1	0	0	-2	0	-1	-3	0	-5	-3
V	-2	-2	-2	2	-2	2	-1	-1	-6	-2

FIG.8

Residue substitutions expected to allow the creation of a disulfide bond

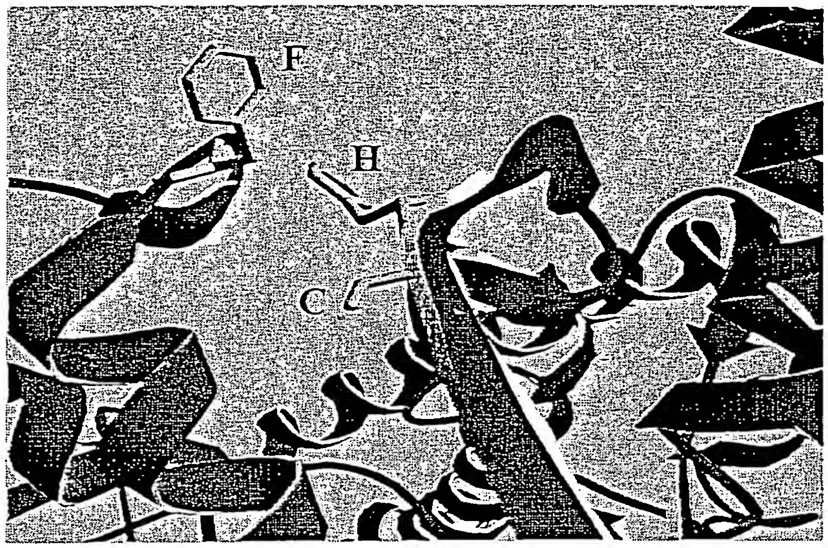

FIG.9A

FIG.9B

Residue substitutions expected to destroy linking interactions

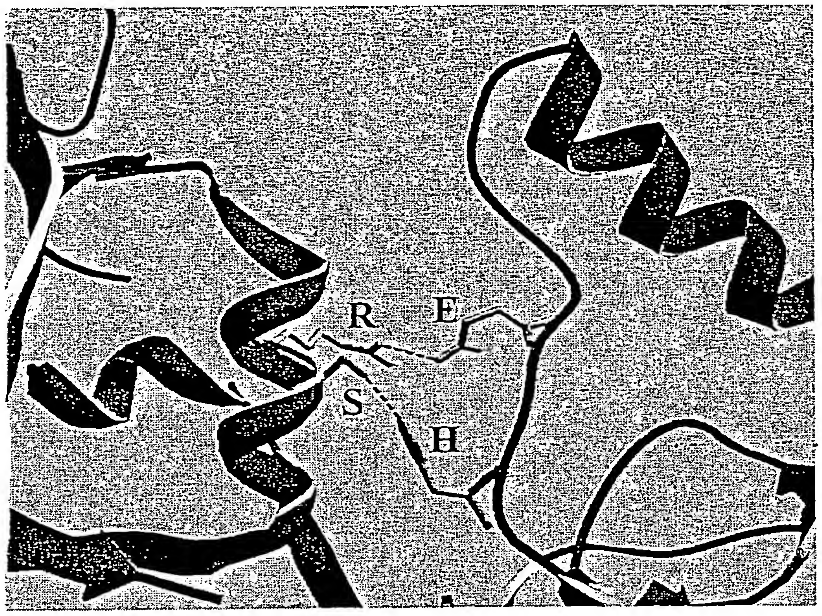

FIG.10A

FIG.10B

Tri-dimensional model of an amphipathic polypeptide

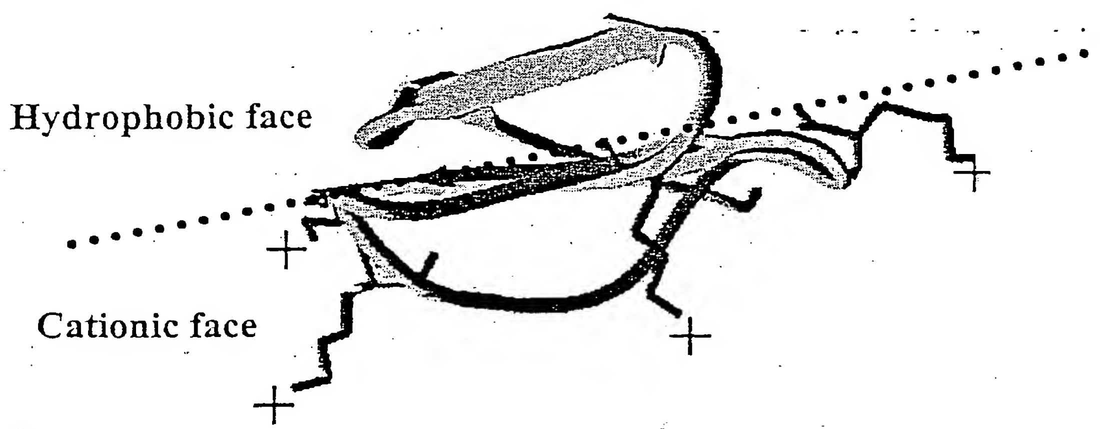

FIG.11

2-D matrix representation of a protein sequence

M V G E S V P A V D Q V L G T S K I Y N E P V N A I W D T I R N W R A H V N P F

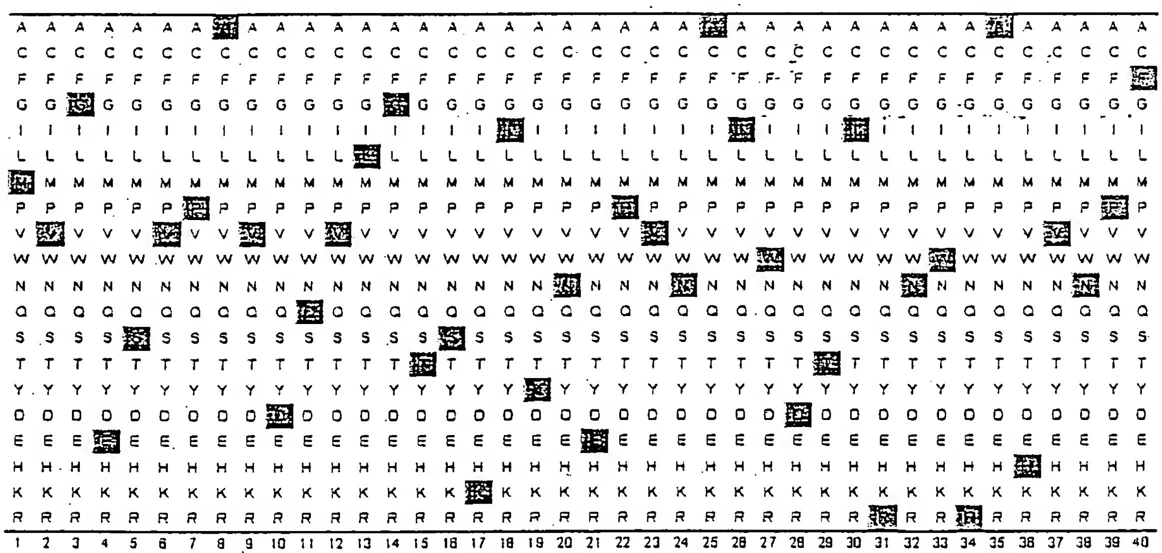

FIG.12

2-D matrix for amphipathic peptide showing K/R scanning: sequential replacement of each residue by either K or R

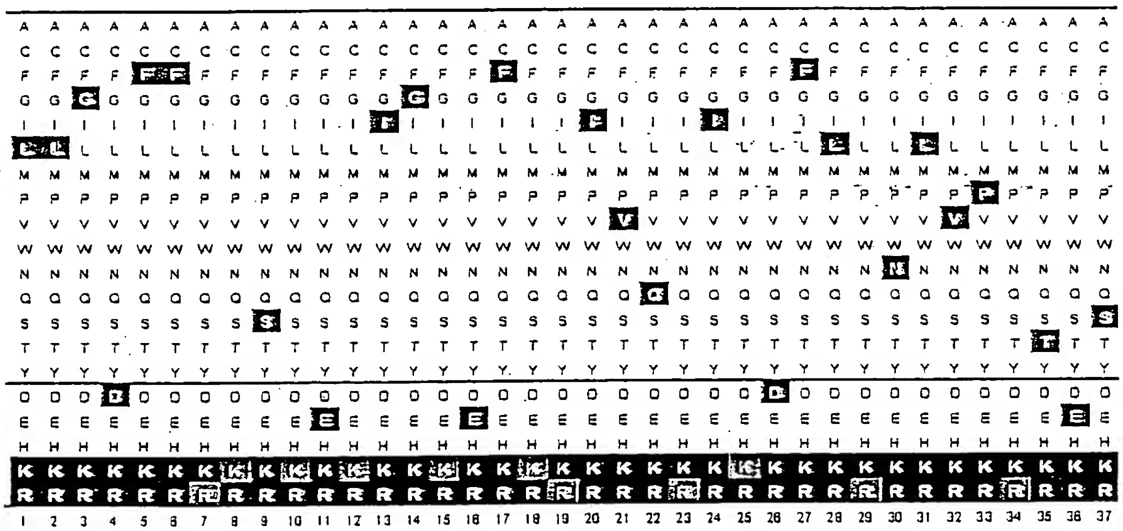

FIG.13A

2-D matrix for K/R scanning on amphipathic polypeptide

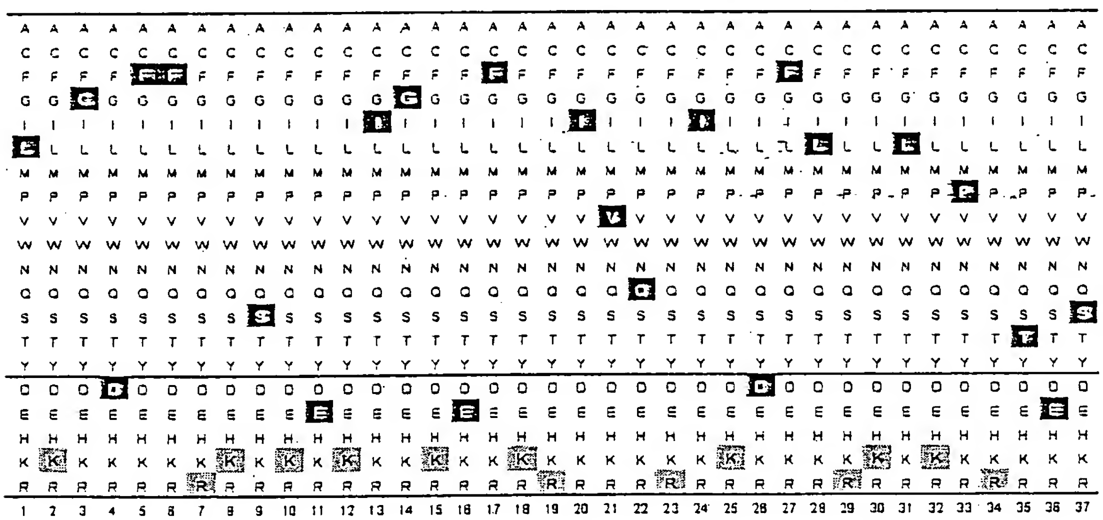

FIG.13B

2-D matrix for LEAD candidates on amphipathic polypeptide

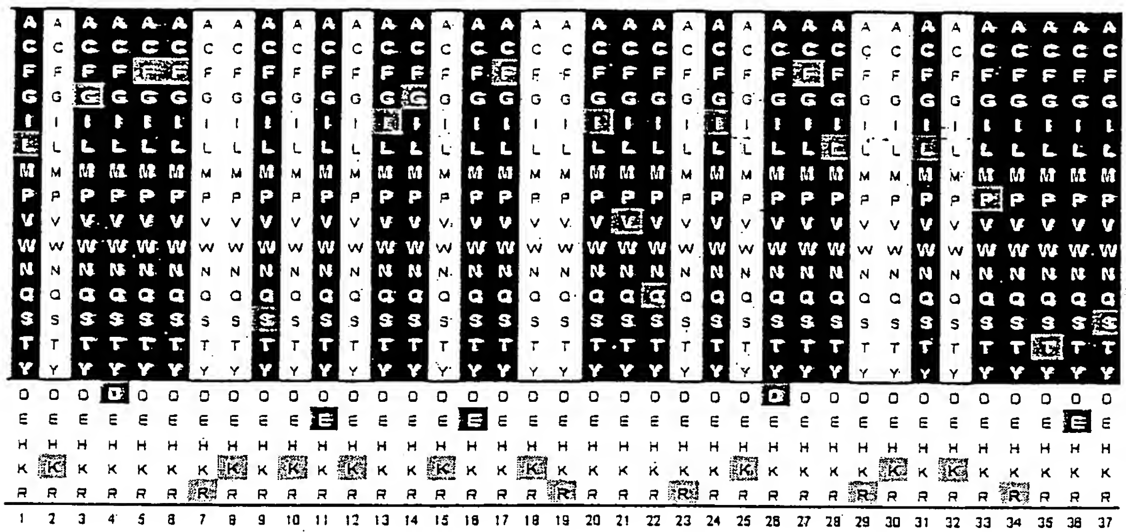

FIG.13C

2-D matrix for optimized amphipathic polypeptide, following both: *i*) K/R scanning (FIG. 13B) and *ii*) mutagenesis (FIG. 13C)

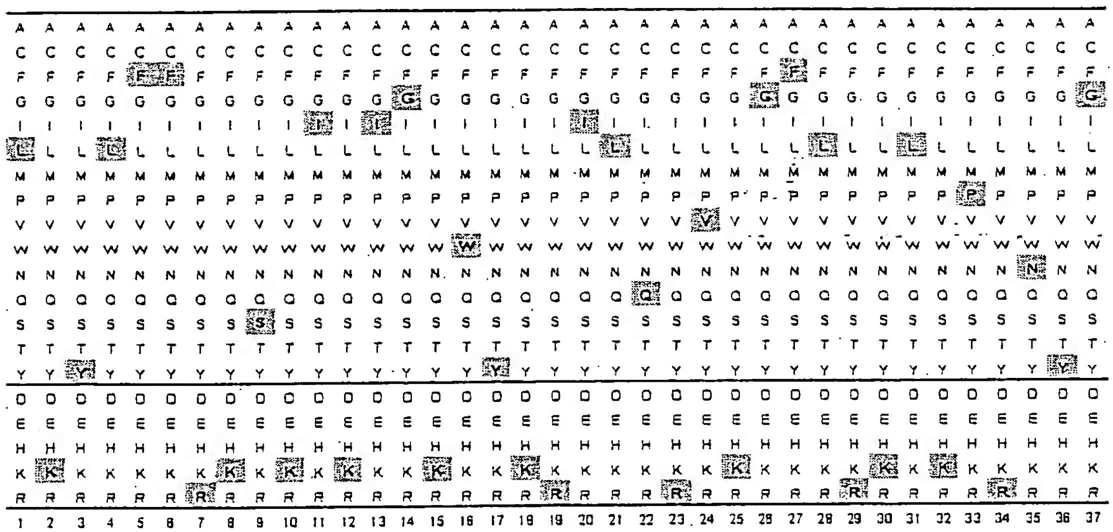

FIG.13D

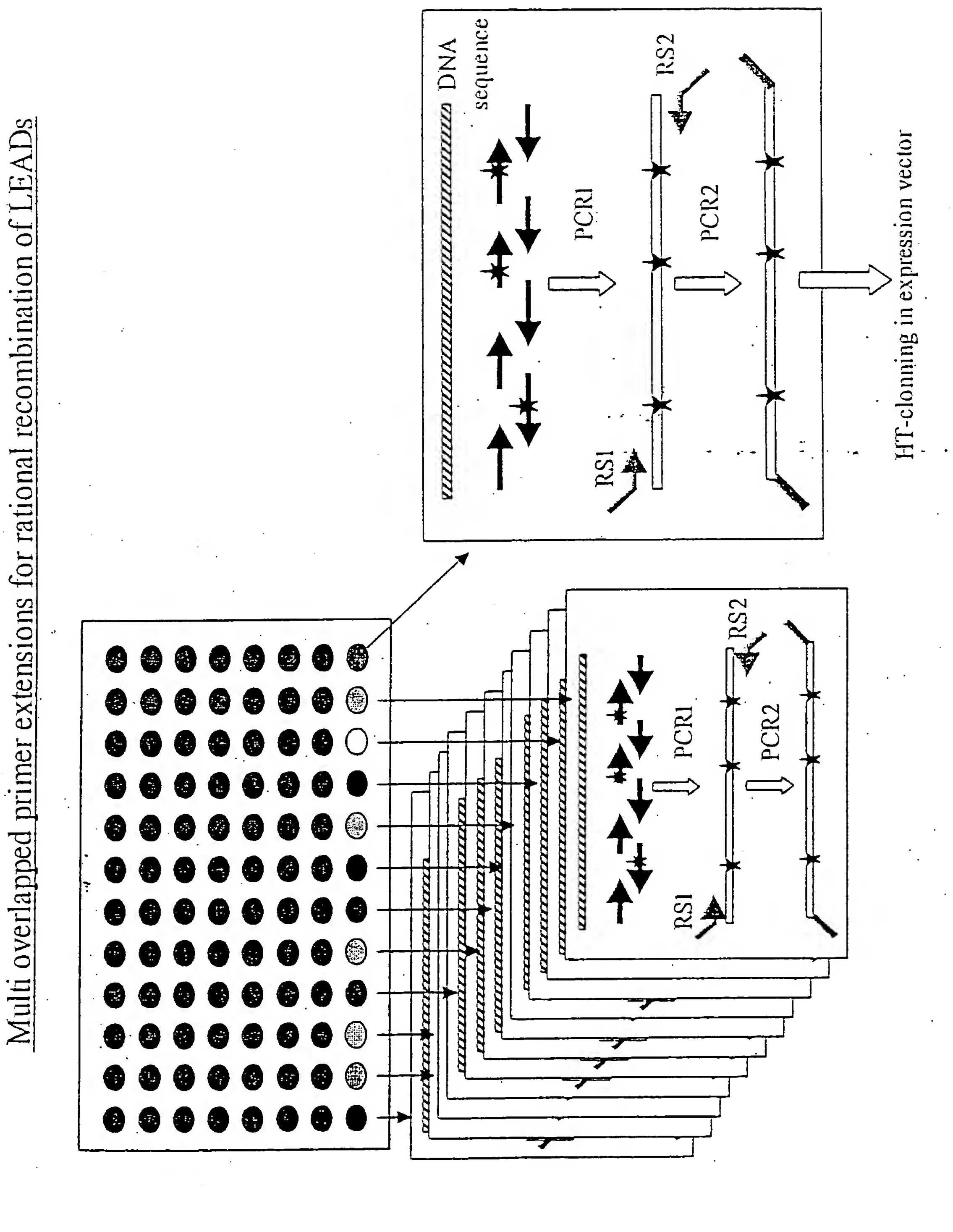
Multi overlapped primer extensions for rational recombination of LEADs
DNA sequence
PCR1
PCR2
RS1
RS2
HT-cloning in expression vector

FIG.14